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Rapid Identification of *Salmonella* Serovars in Feces by Specific Detection of Virulence Genes, *invA* and *spvC*, by an Enrichment Broth Culture-Multiplex PCR Combination Assay

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In order to make a rapid and definite diagnosis of *Salmonella* enteritis in children, an enrichment broth culture-multiplex PCR combination assay was devised to identify *Salmonella* serovars directly from fecal samples. Two pairs of oligonucleotide primers were prepared according to the sequences of the chromosomal *invA* and plasmid *spvC* genes. PCR with these two primers would produce either one amplicon (from the *invA* gene) or two amplicons (from the *invA* and *spvC* genes), depending on whether or not the *Salmonella* bacteria contained a virulence plasmid. The fecal sample was diluted 10- to 20-fold into gram-negative enrichment broth and incubated to eliminate inhibitory compounds and also to allow selective enrichment of the bacteria. One or two amplicons were obtained, the expected result if *Salmonella* bacteria were present. The detection limit of this PCR was about 200 bacteria per reaction mixture. The primers were specific, as no amplification products were obtained with 18 species and 22 isolates of non-*Salmonella* bacteria tested which could be present in the feces or cause contamination. In contrast, when 23 commonly seen *Salmonella* serovars (38 isolates) were tested, all were shown to carry the *invA* gene and seven concomitantly harbored the *spvC* gene of the virulence plasmid. This assay was applied to the diagnosis of *Salmonella* enteritis in 57 children who were suffering from mucoid and/or bloody diarrhea. Of the 57 children, 38 were PCR positive and 22 were culture positive. There were two culture-positive samples that were not detected by PCR. Thus, this PCR assay showed an efficiency of 95% (38 of 40), which is much higher than the 60% (24 of 40) by culture alone. Not only is this method more sensitive, rapid, and efficient but it will cause only an incremental increase in the cost of stool processing, since enrichment cultivation of fecal samples from diarrheal patients using gram-negative enrichment broth is a routine practice for identification in many diagnostic microbiology laboratories. This PCR method, therefore, has clinical application.

Nontyphoid *Salmonella* organisms are the most frequent etiologic agents of bacterial enteritis in children. There are nearly 2,000 *Salmonella* serovars, and for those tested thus far, all seem to contain *inv* genes, which enable the bacteria to invade cells. Also, to date, there are five *Salmonella* serovars known to contain the virulence plasmid that carries the *spv* genes: Typhimurium, Choleraesuis, Dublin, Enteritidis, and Gallinarum-Pullorum (6, 7). Lately, serovars Abortusovis (5) and Sendai have also been found to carry the *spv* genes (unpublished data). Except for Gallinarum-Pullorum which is specific for fowl, the others are the frequently seen etiologic agents of enteritis in humans. Typhimurium is the most common serovar isolated from diarrheal patients, and Choleraesuis, Dublin, and Enteritidis are often isolated from patients with bacteremia (5). Some studies have provided evidence that the virulence plasmid plays a significant role in human disease (5).

The diagnostic method currently in use for *Salmonella* enteritis is culture of the bacteria from the stool samples, a time-consuming and laborious process. Therefore, development of a rapid and sensitive method for the diagnosis of *Salmonella* enteritis is desirable. Several techniques for improving the detection of *Salmonella* serovars in feces, such as the use of a selective culture medium and enzyme-linked immunosorbent assay have been developed (1, 2). However, problems remain with sensitivity and specificity that have limited routine use of these procedures.

PCR technology that allows amplification of a specific fragment of nucleic acid has been used to identify the presence of specific pathogens directly from clinical specimens, such as urine, blood, and cerebrospinal fluid specimens. The major obstacle in the use of fecal samples directly for PCR is the presence of bilirubin and bile salts that are inhibitory to PCR amplification (18, 19). Some reports describe extraction of DNA as a means to eliminate inhibitory substances in feces (3, 19). The DNA extraction procedures devised, however, are labor-intensive and expensive. We herein report a simple, accurate, and fast PCR assay that is a two-step method: the first step is a stool processing step for circumventing the effect of inhibitory compounds as well as selective cultivation of the bacteria, and the second step is a multiplex PCR assay that utilizes the sequences of the *invA* and *spvC* genes of *Salmonella* serovars (4, 6, 7, 13, 14) as targets for amplification of *Salmonella* bacteria in children suffering from enteritis.

Patient selection and sample collection. During the period from 1 November 1994 to 31 June 1995, 57 children suffering from mucoid and/or bloody diarrhea were admitted to the Chang Gung Children's Hospital. Their fecal samples were collected and sent to the microbiology laboratory to determine whether *Salmonella* serovars were present in the fecal samples by the conventional culture method. The samples, for the conventional test, were inoculated into gram-negative (GN) broth (Difco) for enrichment and later plated on xylose-lysine-sodium deoxycholate agar (Difco), *Salmonella-Shigella* agar

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TABLE 1. Synthetic oligonucleotides used as primers for PCR

Primer	Sequence	Gene	Corresponding positions
SPVC-1	ACTCCTTGCACAACCAAATGCGGA	*spvC*	505–528
SPVC-2	TGTCTTCTGCATTTCGCCACCATCA	*spvC*	1052–1075
INVA-1	ACAGTGCTCGTTTACGACCTGAAT	*invA*	104–127
INVA-2	AGACGACTGGTACTGATCGATAAT	*invA*	324–347

(Difco), and *Campylobacter* agar (Difco) for primary selection. After incubation for 12 to 18 h, colonies were purified on the same agar. Identification of *Salmonella* strains was carried out by the biochemical method and the slide agglutination test with specific O antisera (Difco). Some *Salmonella* isolates, when necessary, were further serotyped by the tube agglutination method with H antisera (Difco). *Salmonella* serotypes were classified by the Kauffman-White scheme. In addition to the bacterial cultures, all available fecal specimens were also checked for the presence of rotavirus, a frequent cause of children's diarrhea in this area, with a latex agglutination kit (Orion Diagnostica).

PCR assay. To prepare the fecal samples for PCR assay, fecal samples were diluted 10- to 20-fold to eliminate or minimize the effect of inhibitory compounds, such as bilirubin and bile salts, in the stools. Essentially, 0.5 to 1 ml of the stool was inoculated into 10 ml of GN broth and incubated at 37°C without shaking for 6 h, and the culture obtained was then directly used as the template for PCR.

The oligonucleotide primers for PCR were synthesized according to the published DNA sequences of the *spvC* and *invA* genes, shown to be unique for the *Salmonella* genus and located in the virulence plasmid and chromosome of *Salmonella* serovar Typhimurium, respectively (4, 6, 7, 14, 21). These primer sequences and their corresponding genes are listed in Table 1. PCR with the two primers would produce one or two DNA fragments, depending on the *Salmonella* strains in the stools: one band would be derived from the *invA* gene, and the other band would be derived from the *spvC* gene if a virulence plasmid was present. Therefore, the appearance of at least one band, or two bands if there was a virulence plasmid, would indicate the presence of *Salmonella* bacteria.

The PCR mixture consisted of 5 μl of 10× PCR amplification buffer (Promega), 1.5 mM $MgCl_2$, 200 μM (each) the four deoxyribonucleoside triphosphates, 1 μM (each) of primer pairs, 1.25 U of *Taq* polymerase (Promega), 2 μl of bacterial culture, and double-distilled H_2O that was added to make a total volume of 50 μl. The mixture was overlaid with 50 μl of mineral oil (Sigma) and subjected to 30 PCR cycles in a Programmable Thermal Controller (PTC-100; MJ Research, Inc.). The parameters for the amplification cycles were as follows: denaturation for 30 s at 94°C, annealing of primers for 30 s at 56°C, and primer extension for 2 min at 72°C. Prior to the first cycle, the PCR mixture was incubated for 1 min at 94°C to lyse the bacteria. After the last cycle, the mixture was incubated for 10 min at 72°C, mixed with agarose gel sample buffer with tracking dye, and electrophoresed in a 2% agarose gel. After staining with ethidium bromide, the amplified DNA fragments in the gel were visualized and photographed under UV illumination.

Southern blot hybridization. To confirm that one of the two amplified DNA products was indeed derived from the virulence plasmid, DNA-DNA hybridization was carried out as described below. The PCR products in the gel were transferred to a Zeta-probe membrane (Bio-Rad) and hybridized with the DNA probe by the method of Southern (16). To prepare the probe, the 90-kb virulence plasmid DNA of *Salmonella* serovar Typhimurium strain C5, a wild-type mouse virulent strain (50% lethal dose of <50 bacteria) (12), was first extracted by a modified version (12) of the Kado-Liu method (8) and digested with *Hin*dIII (Promega). The restriction DNA fragments were labeled with [α-^{32}P]dCTP by using the Random Primers DNA Labeling System (Gibco-BRL) (11).

Sensitivity and specificity test. Seeded fecal samples were used to determine the sensitivity of the PCR assay. Strain C5 was used as the seed. One milliliter of a culture of C5 grown overnight (about 10^9 CFU/ml) was serially diluted in 10-fold with GN broth. Before PCR, 1 ml of fecal material was added to 4 ml of GN broth. Two microliters of each diluted sample was mixed with 2 μl of the broth-feces mixture and used as the template for PCR. The fecal material was collected from a healthy child who was not suffering from *Salmonella* infection. Simultaneously, an appropriate volume of each dilution was plated on *Salmonella-Shigella* agar to determine the exact number of viable bacteria in each dilution. To evaluate the specificity of the primers, 18 species of non-*Salmonella* clinical isolates and 23 known *Salmonella* serovars were tested. These bacteria and the numbers of isolates (in parentheses) were as follows: *Shigella sonnei* (1), *Shigella flexneri* (1), *Citrobacter freundii* (1), *Citrobacter diversus* (1), *Bacillus cereus* (1), *Enterobacter cloacae* (1), *Escherichia coli* (3), *Klebsiella pneumoniae* (1), *Proteus mirabilis* (1), *Pseudomonas aeruginosa* (2), *Pseudomonas putida* (1), *Aeromonas hydrophila* (1), *Serratia marcescens* (1), *Staphylococcus aureus* (1), *Staphylococcus epidermidis* (2), *Enterococcus faecalis* (1), *Enterococcus faecium* (1), and *Streptococcus pneumoniae* (1). The *Salmonella* serovars and the numbers of isolates (in parentheses) were as follows: *Salmonella* serovars Typhimurium (5), Schwarzengrund (2), Agona (2), Derby (2), Kaapstad (1), Massenya (1), Abortusovis (1), Clackamas (1), Choleraesuis (4), Infantis (2), Virchow (1), Thompson (1), Muenchen (2), Blockley (1), Newport (1), Typhi (1), Dublin (1), Panama (1), Enteritidis (2), Anatum (2), Weltevreden (1), Gallinarum-Pullorum (2), and Sendai (1).

The sensitivity test with strain C5 indicated that the detection limit of PCR was as low as about 200 bacteria per reaction mixture (50 μl) (Fig. 1). In specificity testing, as expected, all non-*Salmonella* isolates listed above (22 isolates) failed to produce any band, whereas all the *Salmonella* isolates listed above (38 isolates) invariably produced the *invA* amplicon, and seven serovars, Typhimurium, Abortusovis, Choleraesuis, Dublin, Enteritidis, Gallinarum-Pullorum, and Sendai, produced an additional band, the *spvC* amplicon. These results indicate specificity of the primers. This PCR technique was then applied to the clinical samples. Of the 57 patients tested, 40 (70%) were shown to be infected with *Salmonella* organisms detected either by PCR or by culture. Of the 40 positive samples, PCR detected the bacteria in 38 samples (95%), of which 16 samples gave negative results by culture. The remaining two samples gave a positive result by culture and a negative result by PCR. There are two possible reasons for the failure of the PCR to detect the two samples: (i) the presence of unusually high concentrations of inhibitory compounds that were not sufficiently reduced by the level of dilution used and (ii) a bacterial number lower than the detection limit. Since the detection limit of this PCR was as low as about 200 bacteria per reaction mixture as described above, the former was likely the reason for the failure. In this particular test, a sample containing about 20 bacteria gave a positive result when Southern blot hybridization was performed on the PCR product (Fig. 1). This level of detection is consistent with other reports on the use of PCR methodology for the detection of *Salmonella* or other

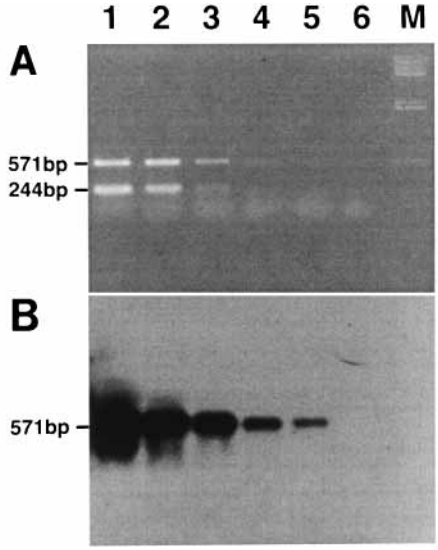

FIG. 1. Sensitivity of PCR and Southern blotting checked with seeded fecal samples. (A) Agarose gel electrophoresis of PCR products. (B) Southern blot. The products in panel A were Southern transferred and probed with the virulence plasmid DNA of strain C5. Lanes: 1 to 6, samples with approximately 2×10^5, 2×10^4, 2×10^3, 2×10^2, 2×10^1, and 2×10^0 C5 organisms, respectively; M, size markers (*Hin*dIII-digested λ DNA). The expected migration positions of the 571-bp and 244-bp amplicons are indicated.

bacteria in feces (3, 18). Thus, the efficiency of this PCR assay in the diagnosis of *Salmonella* enteritis was about 95%, in contrast to 60% by culture alone. Of the 17 patients with negative results by both methods, two were infected with *Campylobacter jejuni* and four were infected with rotavirus. The cause for the remaining 11 was unknown. Of the 38 PCR-positive patients, three also had bacteremia. The amplification of these three samples produced two bands (*spvC* and *invA*) in two samples and one band (*invA*) in the remaining sample. The two isolates with two bands were determined to be serovar Typhimurium, and the other isolate with only one band was determined to be serovar Schwarzengrund. Of the remaining 35 PCR-positive patients who had uncomplicated enteritis, 19 (54%) showed two bands by PCR.

PCR procedures for detecting pathogenic organisms directly from stools have been described by others (3, 20). In such reports, total DNA was extracted after a treatment with proteinase K and phenol-chloroform to eliminate the PCR inhibitors (3, 20). These techniques, however, greatly increase the cost and time; in addition, certain chemicals, such as phenol-chloroform, also inhibit PCR. Later, an immunomagnetic PCR that used magnetic beads with specific antibodies attached for capturing and thus concentrating the organism was devised (19). This procedure was rapid and sensitive, but it was still expensive and limited by the availability of antibodies. Recently, an enrichment culture-PCR combination assay was reported (9, 17). The short enrichment procedure used in these reports prior to PCR is advantageous, since the number of bacteria is substantially increased and the media are selective, relatively inexpensive, and easy to prepare (9, 17). Moreover, the bacteria obtained in the enrichment may be used in the laboratory for other tests such as antibiotic susceptibility and serovar determination. However, the procedures continue to be time-consuming, since extraction of DNA from the enrichment culture is still required for the PCR test (9, 17).

The PCR assay devised in this study, for which DNA extraction is unnecessary, uses GN broth for selective enrichment of the *Salmonella* bacteria whose growth in this broth, furthermore, will not be suppressed by *Proteus*, *Pseudomonas*, and coliform bacteria in the first 6 h of incubation (10). This broth is inexpensive and readily available. To alleviate the interference from the inhibitory compounds in stools, the stool sample is diluted; a 10- to 20-fold dilution was shown to be adequate in this study. It must be emphasized that all specimens used for this study were collected directly from patients. This PCR assay detected nearly all culture-confirmed fecal samples. In addition, a group of 16 patients with evident symptoms of bacterial enteritis had *Salmonella* DNA detected in their feces by PCR but showed negative results by culture. No other etiologic agent was found in any of these patients. The results suggest that this GN broth culture-PCR system performs substantially better than the classical culture technique. The negative results by culture for the 16 patients might be explained by loss of viability of bacteria with specimen handling or a lack of sensitivity of the stool culture system (15). However, the possibility of false-positive PCR results cannot be completely excluded at this time. In view of this possibility, stringent measures are needed when performing this assay to avoid false-positive reactions.

We used two pairs of primers instead of just one in this PCR assay. The advantage of this multiplex PCR is that it can simultaneously identify the *Salmonella* strains that harbor the virulence plasmid, thus facilitating the search for specific etiologic *Salmonella* serovars. This feature is especially advantageous for pediatric patients, since our data showed that most (55%) of the *Salmonella* strains present in the feces derived from children with enteritis harbored a virulence plasmid.

The broth culture-multiplex PCR combination assay for the detection of the *invA* and *spvC* virulence genes thus appears to be a rapid, sensitive, and specific means to simultaneously identify both *Salmonella* serovars and those serovars harboring the virulence plasmid. In addition, since GN broth cultivation of fecal samples from diarrheal patients is a routine practice in diagnostic microbiology laboratories, use of this system will involve only a limited increase in the cost of sample processing.

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